Stomatin-like Protein 2 Is Overexpressed in Cancer and Involved in Regulating Cell Growth and Cell Adhesion in Human Esophageal Squamous Cell Carcinoma

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Abstract **Purpose:** Stomatin-like protein 2 (SLP-2) is a novel and unusual stomatin homologue of unknown functions. It has been implicated in interaction with erythrocyte cytoskeleton and presumably other integral membrane proteins, but not directly with the membrane bilayer. We show here the involvement of SLP-2 in human esophageal squamous cell carcinoma (ESCC), lung cancer, laryngeal cancer, and endometrial adenocarcinoma and the effects of SLP-2 on ESCC cells.

Experimental Design: Previous work of cDNA microarray in our laboratory revealed that SLP-2 was significantly up-regulated in ESCC. The expression of SLP-2 was further evaluated in human ESCC, lung cancer, laryngeal cancer, and endometrial adenocarcinoma by semiquantitative reverse transcription-PCR, Western blot, and immunohistochemistry. Mutation detection of SLP-2 exons was done by PCR and automated sequencing. Antisense SLP-2 eukaryotic expression plasmids were constructed and transfected into human ESCC cell line KYSE450. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, clonogenecity assay, flow cytometry assay, nude mice tumorigenetic assay, and cell attachment assay were done to investigate the roles of SLP-2 gene.

Results: All tumor types we tested showed overexpression of SLP-2 compared with their normal counterparts ($P \leq 0.05$). Moreover, immunohistochemistry analysis of mild dysplasia, severe dysplasia, and ESCC showed that overexpression of SLP-2 occurred in premalignant lesions. Mutation analysis indicated that no mutation was found in SLP-2 exons. KYSE450 cells transfected with antisense SLP-2 showed decreased cell growth, proliferation, tumorigenecity, and cell adhesion.

Conclusions: SLP-2 was first identified as a novel cancer-related gene overexpressed in human ESCC, lung cancer, laryngeal cancer, and endometrial adenocarcinoma. Decreased cell growth, cell adhesion, and tumorigenesis in the antisense transfectants revealed that SLP-2 may be important in tumorigenesis.

Esophageal squamous cell carcinoma (ESCC), the major histologic form of esophageal cancer, is one of the most frequent fatal malignancies in the world, especially in the northern part of China. Human ESCC carcinogenesis is a multistage process involving multifactorial etiology and genetic-environment interactions (1, 2). Genetic changes are speculated to be one of the most important causes for the high prevalence and familial aggregation of ESCC in China (3). It is known that some of the complicated genetic alternations occur frequently in the tumorigenesis of esophagus, such as point mutations of p53 and p16; amplification of cyclin D, c-myc, hst-1, int-2, and epidermal growth factor receptor; as well as allelic loss on chromosomes 3p, 4p, 4q, 5q, 9p21-22, 11p, 11q, 13q, 17p, 18q, and 21q (4–7). However, this is not sufficient to understand the common pathway of tumorigenesis and progression of ESCC. Previous study in our lab has also revealed that a Mendelian autosomal recessive major gene may play a significant role in the etiology of ESCC in a moderate high-incidence area of northern China (1). However, the molecular pathways involved in the pathogenesis of ESCC remain poorly defined. To better understand the molecular mechanisms that underlie the tumor formation and progression, we previously analyzed gene expression profiles of normal and tumor cells systematically (8–10). In addition, previous work on cDNA microarry representing 34,176 clones revealed that SLP-2 was up-regulated over six times in ESCC tissues (11, 12).

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SLP-2 is a novel and unusual member of the stomatin gene superfamily (13–15). The vertebrate homologues identified are SLP-1, SLP-2, and SLP-3 (13, 14, 16). SLP-1 is most abundant in brain and shares many similarities with UNC-24 (13, 14). SLP-3 is specifically expressed in olfactory sensory neurons (13, 16). All of these proteins as well as the stomatin from other species share a characteristic NH_2-terminal hydrophobic domain as well as a consensus stomatin signature sequence that defines the stomatin gene family (13). Similar to other family members, SLP-2 shares the cognate stomatin signature sequence. However, it is the first member of this family to be recognized that lacks an NH_2-terminal hydrophobic domain (13). These features distinguish it from stomatin, SLP-1, and SLP-3. SLP-2 may link stomatin or other integral membrane proteins to the peripheral cytoskeleton and play a role in regulating ion channel conductance or the organization of sphingolipid and cholesterol-rich lipid rafts (13).

In this study, the expression of SLP-2 in four disparate tumor types, ESCC, lung cancer, laryngeal carcinoma, and endometrium adenocarcinoma, was evaluated by semiquantitative reverse transcription-PCR (RT-PCR), Western blot, and immunohistochemistry. To investigate the role of SLP-2 in tumorigenesis, antisense-oriented SLP-2 was stably transfected into KYSE450 cells. Antisense transfection of SLP-2 in KYSE450 cells not only obviously reduced cell growth and proliferation *in vitro* but also inhibited tumor formation and growth *in vivo*. The identification of SLP-2 as a novel cancer-related gene may have implications for understanding tumorigenesis.

Materials and Methods

Tissue specimens. Fresh tissue specimens were taken from patients presented to Cancer Hospital of Chinese Academy of Medical Sciences (Beijing, China) and the First Affiliated Hospital of Anhui Medical University (Anhui province, China) immediately after surgery and stored at −80°C until use. None of the patients had received radiotherapy or chemotherapy before surgery. The study was approved by the Institutional Review Board of Cancer Institute of Chinese Academy of Medical Sciences and Tsinghua University. Tumor tissues were dissected from the resected specimens and the normal tissue block was taken from the distal resection margin. In addition, the sections used for immunohistochemistry were fixed in 4% polyformaldehyde and embedded in paraffin.

Cell culture. ESCC cell lines YES2, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE410, KYSE450, KYSE510, COLO-680N, TE12, and EC9706 were previously established from primary human ESCC patients (12, 17–21). All the cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/μL streptomycin, and 100 μg/μL penicillin (pH 7.2-7.4) in a humidified incubator containing 5% CO_2 at 37°C. KYSE series were generous gifts from Dr. Y. Shimada at Kyoto University (Kyoto, Japan; ref. 17).

Semiquantitative RT-PCR. Total RNA was extracted from frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. The quality of the RNA was assessed by 1% denaturing agarose gel electrophoresis and spectrophotometry. Five micrograms of total RNAs of each sample were reverse transcribed to the first strand of cDNA primed with oligo-$(dT)_{12\text{-}18}$ using Transcriptase SuperScript II Preamplification System for First Strand cDNA kit (Invitrogen). Then 0.5- to 1-μL aliquots of the cDNA were used as template to amplify SLP-2 fragment with primers 5′-GTGACTCTCGACAATGTAAC-3′ (sense) and 5′-TGATCTCATAACG-GAGGCAG-3′ (antisense) and fibronectin 1 fragment with primers 5′-AAGGAGAAGA-CCGGACCAAT-3′ (sense) and 5′-GGCTTGATGG-TTCTCTGGAT-3′ (antisense), both at annealing temperature of 57°C for 27 cycles. The expression of the housekeeping gene *GAPDH* was used as an internal control.

Antibody production and Western blot analysis. Amino acid sequences of SLP-2 were obtained from National Center for Biotechnology Information Protein database and subjected to analysis of secondary structure, hydrophilicity profile, and antigenecity using the program Protean of DNAStar software (DNAStar, Inc., Madison, WI). The polypeptide composed of 13 amino acids (ASLDEELDRVKMS) at the COOH-terminal of SLP-2 (corresponding to amino acids 344-356) was chosen and synthesized, coupled with keyhole limpet hemocyanin at the end of the NH_2-terminal, and emulsified in complete Freund's adjuvant used as antigens for immunization with New Zealand White rabbit and chicken, respectively. Immune response was enhanced with antigens emulsified in incomplete Freund's adjuvant after 14 and 21 days of immunization, respectively. Antibodies were recovered after being subjected to ELISA by Zhuhai Bioinforbody Inc. (Zhuhai, China). The rabbit SLP-2 antibody was then purified by protein A.

For Western blot analysis, cells or tissues were lysed with the buffer [1% SDS, 10 mmol/L Tris-Cl (pH 7.6), 20 μg/μL aprotinin, 20 μg/μL leupeptin, and 1 mmol/L 4-(2-aminoethyl)benzenosulfonyl fluoride]. The protein concentrations were determined using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL). Ten micrograms of protein were separated on 12% of SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with the appropriate primary antibody, anti-SLP-2 antibody (1:1,000 dilution) or mouse monoclonal antibody against fibronectin 1 (1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at 4°C overnight. After washing, the membranes were incubated with secondary antibody at a dilution of 1:3,000 at room temperature for 1 hour. Proteins were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ) and anti-β-actin antibody (Sigma, St. Louis, MO) was used as loading control.

Immunohistochemical staining. Tissue sections were dewaxed with xylene and rehydrated through gradient ethanol into water. After endogenous peroxidase activity was quenched with 3% H_2O_2 for 30 minutes, sections were digested with 0.1% trypsin at 37°C for 30 minutes. After PBS washes, nonspecific antibody binding was blocked by preincubating slides in 10% normal goat nonimmune serum at 37°C for 30 minutes. Sections were incubated with the polyclonal primary antibody against SLP-2 at 1:200 dilution or mouse monoclonal antibody against fibronectin at 1:100 dilution (Santa Cruz Biotechnology) for 2 hours at 37°C or overnight at 4°C. For chicken SLP-2 antibody, sections were incubated with polymer helper at 37°C for 30 minutes. After PBS washes again, sections were incubated with secondary antibody at 1:200 dilution for 30 minutes at 37°C. After PBS washes, sections were developed using 3,3′-diaminobenzidine (Sigma). As for the rabbit SLP-2 antibody or mouse fibronectin 1 antibody, sections were incubated with biotinylated link secondary antibody at 1:200 dilution for 30 minutes and then with horseradish peroxidase–labeled streptavidin for 30 minutes. After PBS washes, sections were developed using 3,3′-diaminobenzidine. Sections were washed in running tap water and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. Negative controls were obtained by omitting the primary antibody.

SLP-2 and fibronectin 1 expression was evaluated as previously described (22, 23). The percentage of SLP-2 positive tumor cells was determined semiquantitatively by assessing the entire tumor section. Each sample was assigned to one of the following categories: 0 (0-4%), 1 (5-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), 2+ (moderate), or 3+, (strong). A final immunoreactive score between 0 and 12 was calculated by multiplying the percentage of positive cells with the staining intensity score. All slides were blindly evaluated for immunostaining without any knowledge of the clinical outcome of other clinical or pathologic data.

DNA extraction and mutation detection. Genomic DNA from human esophageal tissues was extracted by proteinase K digestion and phenol/chloroform extraction as previously described (24). DNA was dissolved in Tris-EDTA buffer and stored at −20°C until use. The sequences of the PCR primers for SLP-2 exons are listed in Table 1. The PCR products amplified with primers were analyzed by electrophoresis on a 3% agarose gel for specificity and further sequenced by automated sequencing. Sequencing results were analyzed by the program SeqMan of DNAStar software (DNAStar).

Preparation of constructs and transfection. Full-length SLP-2 was amplified with the primers 5′-GGCGGTTGGGAAATGCTGGCGCG-3′ and 5′-GTCCCCAGACTCCCTGGCC-3′ by PCR, using cDNA template reverse transcribed from normal esophageal epithelia, and was subcloned into an eukaryotic expression vector (pcDNA3.1/myc-His(−); Invitrogen) in the antisense orientation. Human ESCC cell line KYSE450 was transfected with either the antisense construct or the empty vector using Lipofectamine 2000 (Invitrogen). After G418 (400 μg/μL; Life Technologies, Rockville, MD) screening, the stable clones were identified by semiquantitative RT-PCR and Western blot.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. KYSE450 cells, transfected with either antisense SLP-2 expression vector or empty vector, were digested with trypsin and inoculated in 96-well plates at a concentration of 1×10^3 per well after counting. After incubation at 37°C in a humidified incubator containing 5% CO_2 for 1, 2, 3, 4, 5, 6, and 7 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dissolved in RPMI 1640 at the final concentration of 0.5 mg/μL, was added in a 96-well plate each day. The plates were incubated for an additional 4 hours, then detected at 570 nm on Bio-Kinetics Reader (Bio-Rad, Hercules, CA) after 200 μL of DMSO, instead of RPMI-fetal bovine serum, were added to each well to solubilize the formazan crystals.

Clonogenecity assay. KYSE450 cells, transfected with either antisense SLP-2 expression vector or empty vector, were digested with trypsin and seeded in six-well plates at a concentration of 5×10^2 per well after counting. The plates were incubated at 37°C in a humidified incubator containing 5% CO_2. When the colonies became visible (2-4 weeks), cells were fixed with methanol, stained with Giemsa, and counted.

Flow cytometry assay. Flow cytometry assay was done by propidium iodide staining. KYSE450, empty vector, and antisense SLP-2–transfected cells were grown to 80% to 90% confluence, then digested with trypsin, washed twice with PBS, and fixed overnight at 4°C in 70% ethanol. After washing twice with PBS, cells were incubated with 5 μg/μL propidium iodide and 50 μg/μL RNase A in PBS for 1 hour at room temperature. Flow activated cell sorter analysis was carried out using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) with CellQuest software. A total of 10,000 events were measured per sample.

Experiments in nude mice. Single-cell suspensions of each of the transfectants and parental cells were digested with trypsin and collected. The cell viability was >95% as determined by trypan blue staining. Cells (2×10^6) in a 0.1-μL volume of PBS were inoculated s.c. into the right flank of 4-week-old male BALB/c nude mice (10 for each group). Once palpable tumors were established, tumor volume measurements were taken once a week using calipers along two major axes. At the end of 8 weeks, all mice were sacrificed and the tumor weights were measured.

Cell attachment determination. Cell attachment assay was measured by the CellTiter 96 AQueous One Solution Assay (Promega, Madison, WI) according to the instructions of the manufacturer. Briefly, aliquots of 2×10^4 cells per well were distributed in 96-well plates coated with Matrigel (BD Bioscience, Bedford, MA) in 100 μL of medium. Cell attachment was subsequently determined at 1-, 2-, 4-, 6-, 12- and 24- hour time points after inoculation. At each time point, 20 μL of 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and an electron-coupling reagent (phenazine methosulfate) were added to the medium of the cultured cells and incubated for an additional 1 hour. The relative cell viability was determined with a 490-nm filter. Each experiment was done in triplicate and repeated at least thrice. Relative attachment ratio was calculated as the follows:

$$\text{relative attachment ratio}(\%) = \frac{\text{OD490 of calibrated experimental group}}{\text{OD490 of calibrated parental KYSE450 cells}} \times 100\%$$

where attachment ratio of parental KYSE450 was considered as 100%.

Confocal imaging. Cells were plated on coverslips, fixed in 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100, and blocked for 30 minutes with 3% bovine serum albumin. After simultaneous overnight incubation at 4°C with rabbit anti-SLP-2 immunoglobulin G (1:100) and mouse fibronectin 1 immunoglobulin G (1:100, Santa Cruz Biotechnology) as primary antibodies, the slides were washed in PBS and incubated with the secondary antibodies, FITC-conjugated goat anti-mouse immunoglobulin G (1:50; Molecular Probes, Eugene, OR) and tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit immunoglobulin G (1:50; Molecular Probes). Nuclei were counterstained with 1 μg/μL 4′,6-diamidino-2-phenylindole (Sigma). Slides were mounted with Mowoil and examined with Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). Series of images were processed and analyzed with the accompanying software package.

Statistical analysis. Statistical analysis was done using the SPSS statistical software (SPSS, Inc., Chicago, IL). The correlation between SLP-2 expression and clinicopathologic characteristics or variables was analyzed using Spearman's correlation analysis. χ^2 test was done for

Table 1. Primers used for mutation detection of SLP-2 exons and conditions of PCR

Exon	Direction	Primer sequence (5′-3′)	Annealing temperature (°C)	10% DMSO	Production size (bp)
E1, E2	Forward	TAACGCTGATTGGGTGAGTG	66	+	613
	Reverse	AGGGATTGGTCATCTGGCTG			
E3, E4	Forward	AGTGACACCTCAGCCTTCTG	67	—	538
	Reverse	TGAGATGGACACGGATAGTG			
E5	Forward	ACTATCCGTGTCCATCTCAG	55	—	158
	Reverse	GTCTTCAAACCCTAACTCTG			
E6, E7	Forward	CAGTGTAGGCCCTTGATAGG	65	—	592
	Reverse	GGACAGAGCTTGCTTGACCC			
E8, E9	Forward	ACTTCATGGGTCAAGCAAGC	66	+	478
	Reverse	GTGCTGGGTCCTCAGAGAAGC			
E10	Forward	CCATTGGTAGGCTGGGCTGC	66	+	346
	Reverse	TCTGGTTTGCCACTGGTGAG			

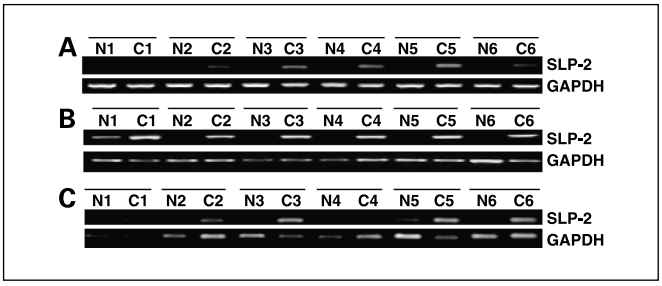

Fig. 1. Overexpression of SLP-2 in human lung cancer, laryngeal cancer, and endometrial cancer by semiquantitative RT-PCR. SLP-2 is overexpressed in lung cancer (*A*), laryngeal cancer (*B*), and endometrial cancer (*C*). *GAPDH* is used as an internal control. N, normal tissue; C, patient-matched tumor tissue.

comparison unless particular test was notified. Data from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, clonogenecity assay, and tumorigenecity assay were presented as means ± SD. $P < 0.05$ was considered statistically significant.

Results

Overexpression of SLP-2 in different kinds of tumors. Previous work on cDNA microarry and RT-PCR revealed that SLP-2 was up-regulated over six times in ESCC (8, 11, 12). Semiquantitative RT-PCR of SLP-2 expression in patient-matched normal and tumor epithelium from different individuals with lung cancer, laryngeal cancer, and endometrial adenocarcinoma was done to further evaluate the differential expression in different kinds of human tumors. As it was shown in Fig. 1, overexpression of SLP-2 was observed in cancer tissues of different kinds of human tumors (Table 2).

To confirm the overexpression of SLP-2 in cancer tissues at the protein level, amino acid sequences of SLP-2 obtained from National Center for Biotechnology Information Protein database were analyzed using the program Protean; secondary structure and hydrophilicity profile results were the same as reported (13). Polypeptide composed of 13 amino acids (ASLDEELDRVKMS) was chosen as antigens for immunization with rabbit and chicken (25). SDS-PAGE and ELISA indicated that both antibodies against SLP-2 were free of contaminating proteins (>90% purity) and high in titer (1:1,000-1:10,000). Immunoblot analysis of SLP-2 protein expression in patient-matched normal and tumor epithelium from different individuals with ESCC and lung cancer was done and rabbit polyclonal antibody against SLP-2 was fit for Western blotting. Using the available antibody, SLP-2 was also found to be up-regulated in 72% of ESCC tissues (13 of 18) and 68% of lung tissues (13 of 19), respectively (Fig. 2; Table 2). Western blotting with the commercially available antibody against SLP-2 (Proteintech Group, Inc., Chicago, IL) also got similar results (data not shown).

Immunohistochemistry was also carried out to reveal the expression of SLP-2 in ESCC, lung cancer, laryngeal cancer, and endometrial adenocarcinoma, and both antibodies could be used for detection of the antigen in tissues. However, the specificity of the chicken polyclonal antibody is better than the rabbit one. Using chicken polyclonal antibody, SLP-2 was found up-regulated in 92% of ESCC samples (86 of 93, $P \leq 0.0001$), 76% of lung cancer samples (26 of 35, $P < 0.05$) with 89% of squamous cell carcinoma (16 of 18, $P < 0.05$) and 59% of adenocarcinoma (10 of 17, $P < 0.05$), 90% of laryngeal carcinoma (76 of 84, $P \leq 0.001$), and 77% of endometrium adenocarcinoma (10 of 13, $P < 0.05$) compared with their normal counterparts (Fig. 3; Table 2). Strong positive staining was presented in plasma membrane cytoplasm of different kinds of human tumors (Fig. 3). Immunohistochemical staining with rabbit polyclonal antibody against SLP-2 and the commercially available antibody against SLP-2 (Proteintech Group) also got similar results (data not shown). However, no correlation was found between SLP-2 expression and the pathologic characteristics (Table 3).

Overexpression of SLP-2 in premalignant lesions of ESCC development. To determine whether the overexpression of SLP-2 occurred in premalignant lesions of ESCC, immunohistochemical staining was done with mild dysplasia, severe dysplasia, and ESCC. The expression of SLP-2 in normal epithelial cells was always negative whereas positive staining was found in mild dysplasia, severe dysplasia, and squamous cell carcinoma including keratin pearl, which indicated that overexpression of SLP-2 is an early event in esophageal cancer development. Moreover, its expression was significantly increased as ESCC tumorigenesis progressed (Fig. 3).

Mutation detection of SLP-2 exons. Mutation detection of SLP-2 exons was done using PCR and automated sequencing with 30 patient-matched tissues. No mutation was found within the open-reading frame of SLP-2 after sequencing results were aligned by the procedure SeqMan of DNAStar software (DNAStar; data not shown).

Table 2. Overexpression of SLP-2 in human ESCC, lung cancer, laryngeal cancer, and endometrial adenocarcinoma

Tumor type	Ratio of SLP-2 overexpression			
	RT-PCR	Western blot	Immunohistochemistry	*P*
ESCC	40:54 (74%)	13:18 (72%)	86:93 (92%)	≤0.0001*
Lung cancer	38:49 (78%)	13:19 (68%)	26:35 (76%)	<0.05*
Squamous cell carcinoma	20:24 (83%)	7:10 (70%)	16:18 (89%)	<0.05*
Adenocarcinoma	16:25 (64%)	6:9 (67%)	10:17 (59%)	<0.05*
Laryngeal carcinoma	30:36 (83%)	ND	75:84 (89%)	≤0.001*
Endometrium adenocarcinoma	20:30 (67%)	ND	10:13 (77%)	<0.05*

*$P \leq 0.05$, versus normal tissues. ND, not detected.

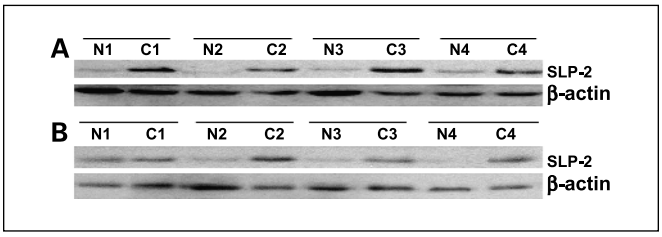

Fig. 2. Overexpression of SLP-2 in ESCC and lung cancer detected by Western blot. SLP-2 is overexpressed in ESCC (*A*) and lung cancer (*B*) compared with normal counterparts. β-Actin was used as a loading control.

Screening of ESCC cell lines and positive transfectants. Semiquantitative RT-PCR and Western blot analysis of 12 ESCC cell lines showed that KYSE30, KYSE410, KYSE450, and TE12 had higher expression of endogenous SLP-2 (Fig. 4A). To investigate the role of SLP-2 gene in human ESCC cells, KYSE450 cells were chosen for antisense transfection and further study. After G418 screening, two antisense stable clones (AS-3 and AS-4) and one empty vector clone were identified and chosen for the following studies (Fig. 4B).

***Transfectants with antisense SLP-2 inhibits cell growth* in vitro *and tumor growth* in vivo.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and clonogenecity assay showed that cell growth and proliferation of antisense stable clones (AS-3 and AS-4) was slower than that of KYSE450 or empty vector cells under normal culture conditions (Fig. 4C and D). As inhibition of cell growth and proliferation can be the consequence of an induction either of apoptosis or of cell cycle arrest, flow cytometry assay was done. As shown in Fig. 4E, no apoptosis was detected in antisense SLP-2–transfected cells whereas antisense SLP-2 constructs resulted in an arrest at S-phase by ~11% to 13% compared with empty vector controls (Fig. 4E). In addition, we have previously observed that antisense transfection of SLP-2 caused S-phase arrest in another ESCC cell line, TE12 (12).

Four groups of nude mice inoculated with antisense SLP-2, empty vector, or KYSE450 cells were sacrificed at the end of 8 weeks. The data showed that tumors from antisense SLP-2 transfectants in nude mice grew more slowly than that from KYSE450 or empty vector cells, which was consistent with the cell proliferation results *in vitro*. The weights of tumors from antisense SLP-2 transfectants were significantly less than that from KYSE450 or empty vector cells whereas no obvious difference was found between parental cells and the vector control cells (Fig. 4F).

Transfectants with antisense SLP-2 inhibits cell attachment. Cell attachment was determined by the CellTiter 96 AQueous One Solution Assay. The result showed that cell adhesion of antisense transfectants (AS-3 and AS-4) was decreased sharply than that of parental KYSE450 or empty vector cells after 4 hours of inoculation ($P < 0.05$) whereas there was no significant difference between parental KYSE450 cells and empty vector cells. With time prolongation, the adhesive ability of antisense transfectants increased although adhesive force of antisense transfectants was still weaker than that of parental KYSE450 or empty vector cells (Fig. 5A).

Cell adhesion played important roles in maintaining normal functions of cells including cellular organization and structure, proliferation, survival, metabolism, and gene expression. Alterations in cell adhesion were in the control of cell behavior during invasion and metastasis of malignant cancer cells. Several cellular adhesion molecules such as fibronectin 1, laminin B1, β-catenin, E-cadherin, intercellular adhesion molecule-1, and E-selectin were detected in antisense transfectants (AS-3 and AS-4) and parental KYSE450 or empty vector cells by semiquantitative RT-PCR and Western blot. Only fibronectin 1 was found drastically decreased in antisense transfectants compared with parental KYSE450 cells and empty vector cells (Fig. 5B) whereas no differences were found for other adhesive molecules we tested. Fibronection 1 and SLP-2 expression was evaluated simultaneously in 25 pairs of patient-matched ESCC tissues by semiquantitative PCR and in 18 pairs of ESCC tissues which were from the above 25 paired samples by Western blot (Fig. 5C). After band quantification with Leica QWin image analysis and image processing software, correlation between fibronection 1 and SLP-2 expression was done using Pearson's correlation analysis. The results suggested that there was a good correlation between fibronection 1 and SLP-2 expression ($r_P = 0.661$, $P = 0.000$). Immunohistochemical staining was also done to analyze the correlation between fibronection 1 and SLP-2

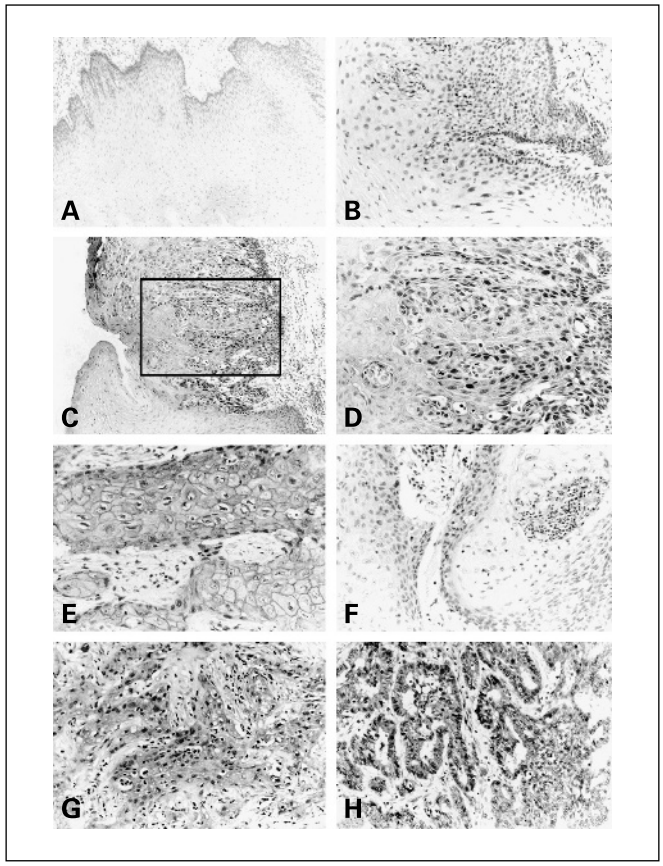

Fig. 3. Immunohistochemical staining analysis of SLP-2 expression in normal epithelia, mild dysplasia, severe dysplasia, ESCC, as well as lung adenocarcinoma and endometrium adenocarcinoma. *A,* negative staining of SLP-2 in normal esophageal epithelia (× 100). *B,* positive staining was seen in mild dysplasia of esophagus (× 200). *C, D,* and *E,* strong positive staining of SLP-2 was found in cell membrane of severe dysplasia of esophagus (*C*, × 100; *D*, × 200) and in plasma membrane and cytoplasm of ESCC (*E*, × 200). *F,* negative control of ESCC (× 200). The primary antibody was replaced by nonimmune serum. *G* and *H,* strong positive staining was found in plasma membrane of lung adenocarcinoma (*G*, × 200) and endometrium adenocarcinoma (*H*, × 200). All sections were counterstained with hematoxylin.

Table 3. Relationship between clinical data and SLP-2 expression by immunohistochemistry

Tumor type	Total cases	Female/male	Mean age (range), y	Differentiation				LNM			Invasion		
				WD	MD	PD	*P*	+	–	*P*	ML	WE	*P*
ESCC	93	15/78	59 (36-79)	19	52	12	0.265	31	54	0.090	25	31	0.407
Lung cancer	35	9/26	61 (37-79)	14	10	10	0.849	20	12	0.898	—	—	—
Squamous cell carcinoma	18	5/13	62 (37-79)	6	6	5	0.654	8	7	0.853	—	—	—
Adenocarcinoma	17	8/9	60 (39-76)	8	4	5	0.868	12	5	0.371	—	—	—
Laryngeal carcinoma	84	16/68	61 (39-84)	46	32	5	0.887	37	29	0.363	—	—	—
Endometrial adenocarcinoma	13	13	56 (39-67)	6	5	2	0.447	7	6	0.215	—	—	

NOTE: Statistically significant at $P \leq 0.05$; —, indicates data not available.
Abbreviations: LNM, lymph node metastasis; WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; ML, molecular leyer; WE, whole epithelium.

expression in ESCC tissue arrays including 50 pairs of patient-matched samples (Supplementary data). Correlation analysis using Spearman's correlation analysis indicated that there was a good correlation between fibronection 1 and SLP-2 expression ($r_s = 0.297$, $P = 0.004$). Confocal imaging was further done to analyze the localization of SLP-2 and fibronectin 1, and the result showed that the two proteins colocalized at the membrane (Fig. 5D).

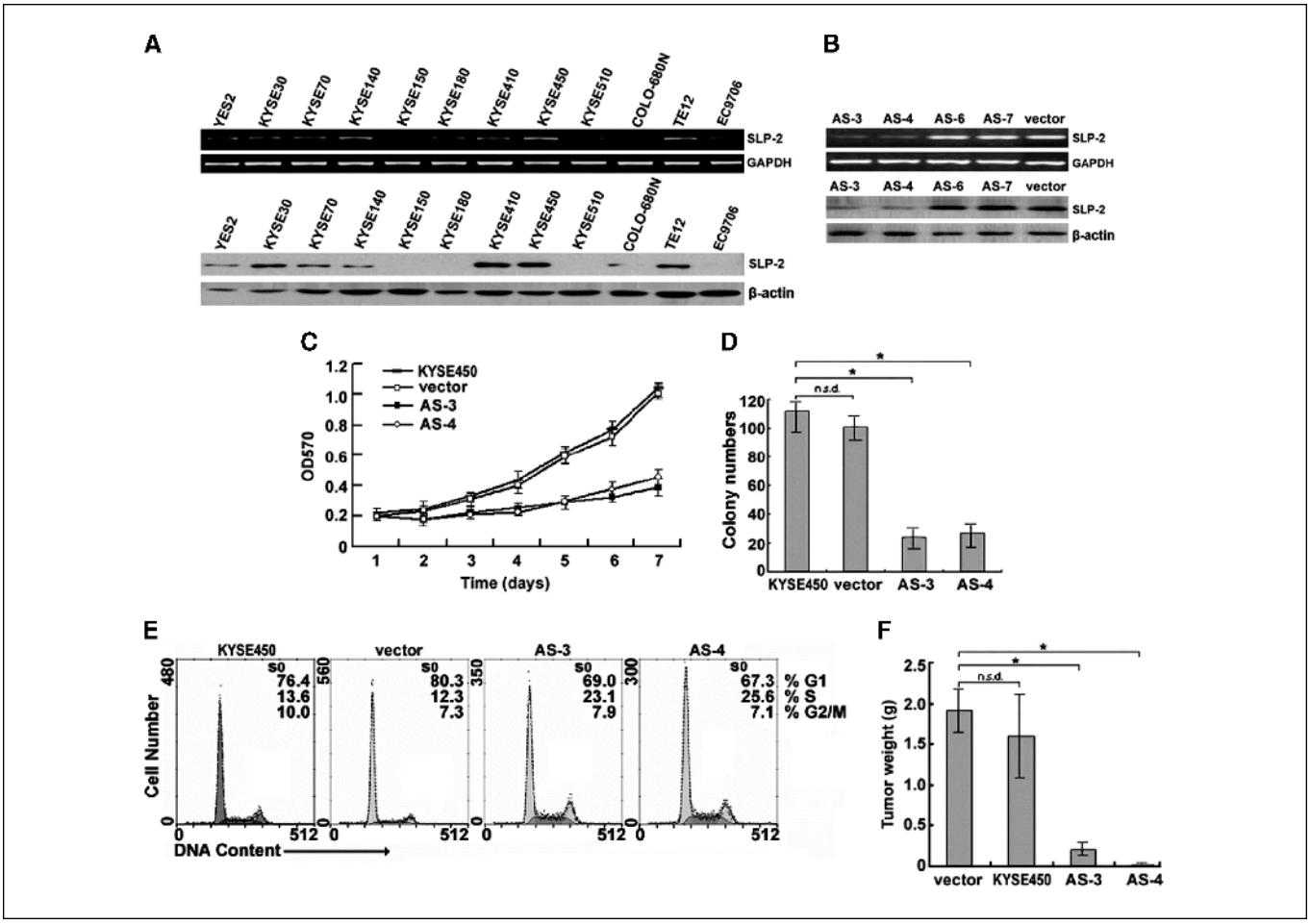

Fig. 4. Expression of SLP-2 in ESCC cell lines and the effects of SLP-2 on tumor growth and tumorigenecity. *A,* ESCC cell lines with endogenous SLP-2 expression were screened by semiquantitative RT-PCR and Western blot. *B,* positive clones transfected with antisense SLP-2 were identified by semiquantitative RT-PCR and Western blot (AS-3, AS-4, AS-6, and AS-7 were different individual transfectants), AS-3 and AS-4 showed obviously reduced SLP-2 expression and were chosen for further analysis. *C,* growth curve by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; antisense transfectants AS-3 and AS-4 showed obviously reduced cell growth compared with controls (vector and KYSE450). *D,* clonogenecity assay showing that antisense transfectants AS-3 and AS-4 had lower clonogenecity as compared with controls (vector and KYSE450). *E,* flow cytometry analysis revealed that antisense transfectants AS-3 and AS-4 caused S-phase arrest compared with controls (vector and KYSE450). *F,* tumorigenecity of antisense clones AS-3 and AS-4 compared with controls (vector and KYSE450). Tumor weight from KYSE450 cells, empty vector transfectants, and cell clones AS-3 and AS-4 cells were shown as mean ± SD of three independent experiments. *, $P < 0.001$; n.s.d., no significant difference. AS-3 and AS-4, different individual transfectants with antisense SLP-2; vector, transfectants with empty vector; KYSE450, parental KYSE450.

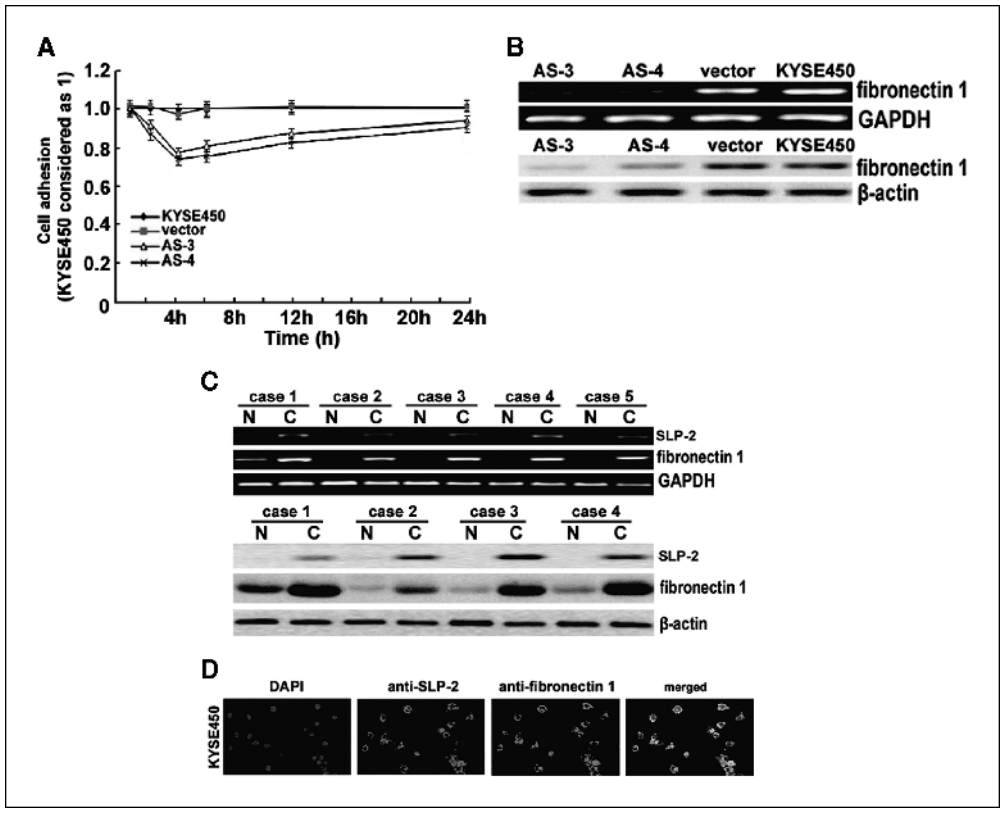

Fig. 5. Correlation analysis between fibronectin 1 and SLP-2 expression. *A,* cell attachment assay. Cell adhesion of antisense transfectants (AS-3 and AS-4) was decreased sharply compared with controls (vector and KYSE450) after 4 hours of inoculation ($P < 0.05$) whereas there was no significant difference between parental KYSE450 cells and empty vector cells. *B,* expression of fibronectin 1 in different individual transfectants detected by semiquantitative RT-PCR and Western blot after 4 hours of inoculation. *C,* expression of fibronection 1 and SLP-2 in patient-matched ESCC tissues by semiquantitative PCR and Western blot. *D,* colocalization of SLP-2 and fibronectin 1 by confocal imaging. AS-3 and AS-4, different individual transfectants with antisense SLP-2; vector, transfectants with empty vector; KYSE450, parental KYSE450 cells.

Discussion

Tumorigenesis is a complex and multistage process with many genes involved. To develop rational approaches to the diagnosis and treatment of cancer depends on identifying and understanding the molecular mechanisms that underlie tumor formation and progression. In this aspect, studies that seek to identify dysregulated genes and proteins in neoplasm are critical. Thus, research efforts aimed at systematically identifying the gene and protein expression profiles of normal and tumor cells are critically needed. Previous work on cDNA microarry to analyze gene expression profiles of normal and tumor cells systematically was done in our laboratory (8–10), and SLP-2 was first identified as an up-regulated gene in ESCC tissues (11, 12). The present study is, to our knowledge, the first to identify SLP-2 as a novel cancer-related gene and to examine the potential role of SLP-2 in tumorigenesis.

Our findings show that SLP-2 was associated with the different stages of tumor progression from normal tissue to premalignant and malignant lesions of esophagus. Previous studies have shown that squamous epithelial dysplasia is a precancerous lesion of ESCC (9). Usually, squamous epithelial dysplasia occurs about 10 years before the appearance of ESCC. Overexpression of SLP-2 in the early stage of ESCC suggests that SLP-2 may be fundamentally important in human tumorigenesis. The elucidation of mechanisms governing malignant changes from dysplasia to ESCC at the molecular level may provide a further understanding of tumorigenesis, as well as new approaches to the strategy of early prevention and treatment of ESCC. In addition, an effective nonsurgical therapy for patients with early ESCC, endoscopic laser therapy, has been designed and may play a role in early ESCC treatment. Thus, early detection of ESCC becomes extremely meaningful. The overexpression of SLP-2 in premalignant lesions indicated that it might serve as a marker for early detection of ESCC.

SLP-2 was first identified to be overexpressed in human ESCC, lung cancer, laryngeal cancer, and endometrial adenocarcinoma, which showed that SLP-2 overexpression was very common in cancer development. However, the status of SLP-2 expression in other kinds of human tumors still remained unclear. The overexpression of SLP-2 in many kinds of human tumors and the inhibition of tumorigenesis of antisense construct made it more likely to be a potential oncogene. We further transfected sense-oriented SLP-2 to human fibroblast cell NIH 3T3. Unfortunately, no malignant transformation was found. As we know, oncogenes would confer to tumorigenic potential in athymic nude mice. However, in many cases, a single oncogene was weakly sufficient for malignant transformation of immortalized NIH 3T3 cells. Cotransfection of one oncogene with another would show a synergistic action between the two oncogenes in the transformation of NIH 3T3 fibroblasts (26). Although there were many oncogenes identified by now, this study will add more information on understanding the molecular mechanisms of tumorigenesis and will shed light on the development of better treatment and better diagnostic and preventive approaches to cancer.

Most cells adhere to their neighbors and to the extracellular matrix, a fibrillar meshwork surrounding or underlying most cells in the body. During embryologic development,

cell adhesion is important for the correct movements of cells modeling the embryo. In the adult, appropriate cell adhesion is necessary for numerous physiologic processes and can be deranged in many diseases, including cancer. During cell culture, we found that cells transfected with antisense SLP-2 were more easily digested by trypsin than parental KYSE450 cells and empty vector cells (12). We then speculated that SLP-2 may have some relation with cell adhesion and subsequently carried out the cell attachment assay to determine cell adhesion ability by the CellTiter 96 AQueous One Solution Assay. This assay was a nonradioactive procedure that measured metabolic function that directly correlated with living cell numbers. Transfectants with antisense SLP-2 reduced cell attachment, revealing that SLP-2 might be involved in cell adhesion. To further characterize the cell adhesion molecules involved in the process, the expression changes of some adhesion molecules, such as fibronectin 1, laminin B1, β-catenin, E-cadherin, and intercellular adhesion molecule-1 and E-selectin, were tested by RT-PCR and Western blot analysis. The results showed that one particularly important extracellular matrix protein, fibronectin 1, was sharply down-regulated in transfectants with antisense SLP-2 and colocalized at membrane with SLP-2. Fibronectins comprised a group of closely related proteins, all encoded by a single gene, and they promoted cell adhesion and cell migration and affected many other cellular processes (27). Colocalization of SLP-2 and fibronectin 1 at membrane indicated that SLP-2 might serve as transmembrane linkers between the extracellular matrix outside and the cytoskeleton and signaling systems inside cells.

The etiology of increased SLP-2 expression in human cancers is unknown. Possible mechanisms include point mutation, gene amplification, gene rearrangement, and insertion of strong promoter or enhancer. Epigenetic modifications including demethylation and deacetylation may also be responsible. Mutation detection of SLP-2 exons with 30 patient-matched ESCC tissues did not reveal any mutation within the open reading frame of SLP-2. Further studies to investigate the mechanism of SLP-2 overexpression are currently under way. Antisense transfection of SLP-2 gene led to the arrest of S phase and the inhibition of cell growth, proliferation, and tumor growth. These findings revealed that up-regulation of SLP-2 causes the cancer cells to become hyperproliferative and overexpression of SLP-2 may contribute to the malignant phenotype of ESCC.

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References

1. Zhang W, Bailey-Wilson JE, Li W, et al. Segregation analysis of esophageal cancer in a moderately high-incidence area of northern China. Am J Hum Genet 2000;67:110–9.
2. Su H, Hu N, Shih J, et al. Gene expression analysis of esophageal squamous cell carcinoma reveals consistent molecular profiles related to a family history of upper gastrointestinal cancer. Cancer Res 2003;63:3872–6.
3. Chang-Claude J, Becher H, Blettner M, Qiu S, Yang G, Wahrendorf J. Familial aggregation of oesophageal cancer in a high incidence area in China. Int J Epidemiol 1997;26:1159–65.
4. Chen BS, Wang MR, Xu X, et al. Transglutaminase-3, an esophageal cancer-related gene. Int J Cancer 2000;88:862–5.
5. Metzger R, Schneider PM, Warnecke-Eberz U, Brabender J, Holscher AH. Molecular biology of esophageal cancer. Onkologie 2004;27:200–6.
6. Mayama T, Fukushige S, Shineha R, Nishihira T, Satomi S, Horii A. Frequent loss of copy number on the long arm of chromosome 21 in human esophageal squamous cell carcinoma. Int J Oncol 2000;17:245–52.
7. Montesano R, Hollstein M, Hainaut P. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. Int J Cancer 1996;69:225–35.
8. Luo A, Kong J, Hu G, et al. Discovery of Ca^{2+}-relevant and differentiation-associated genes down-regulated in esophageal squamous cell carcinoma using cDNA microarray. Oncogene 2004;23:1291–9.
9. Lu J, Liu Z, Xiong M, et al. Gene expression profile changes in initiation and progression of squamous cell carcinoma of esophagus. Int J Cancer 2001;91:288–94.
10. Zhi H, Zhang J, Hu G, et al. The deregulation of arachidonic acid metablism-related genes in human squamous cell carcinoma. Int J Cancer 2003;106:327–33.
11. Zhang LY, Wang T, Ding F, Liu ZM, Liu ZH, Li YD. Differential expression and bioinformatics analysis of SLP-2 gene in esophageal squamous cell carcinoma. World Chin J Digestol 2004;12:1517–21.
12. Zhang LY, Ding F, Liu ZM, Li WD, Liu ZH, Li YD. Effect of stomatin-like protein 2 (SLP-2) gene on growth and proliferation esophageal squamous cell carcinoma cell line TE12. Ai Zheng 2005;24:155–9.
13. Wang Y, Morrow JS. Identification and characterization of human SLP-2, a novel homologue of stomatin (Band 7.2b) present in erythrocytes and other tissues. J Biol Chem 2000;275:8062–71.
14. Seidel G, Prohaska R. Molecular cloning of hSLP-1, a novel human brain-specific member of the band 7/MEC-2 family similar to *Caenorhabditis elegans* UNC-24. Gene 1998;225:23–9.
15. Sprenger RR, Speijer D, Back JW, De Koster CG, Pannekoek H, Horrevoets AJ. Comparative proteomics of human endothelial cell caveolae and rafts using two-dimensional gel electrophoresis and mass spectrometry. Electrophoresis 2004;25:156–72.
16. Goldstein BJ, Kulaga HM, Reed RR. Cloning and characterization of SLP-3: a novel member of the stomatin family expressed by olfractory receptor neurons. J Assoc Otolaryngol 2003;4:74–82.
17. Shimada Y, Imamura M, Wagata T, Yamaguchi N, Tobe T. Characterization of 21 newly established esophageal cancer cell lines. Cancer 1992;69:277–84.
18. Kanda Y, Nishiyama Y, Shimada Y, et al. Analysis of gene amplification and overexpression in human esophageal-carcinoma cell lines. Int J Cancer 1994;58:291–7.
19. Raida M, Sarbia M, Clement JH, Adam S, Gabbert HE, Hoffken K. Expression, regulation and clinical significance of bone morphogenetic protein 6 in esophageal squamous-cell carcinoma. Int J Cancer 1999;83:38–44.
20. Katoh M. Expression of human SOX7 in normal tissues and tumors. Int J Mol Med 2002;9:363–8.
21. Han Y, Wei F, Xu X, et al. Establishment and comparative genomic hybridization analysis of human esophageal carcinomas cell line EC9706. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2002;19:455–7.
22. Sarbia M, Loberg C, Wolter M, et al. Expression of bcl-2 and amplification of c-myc are frequent in basaloid squamous cell carcinomas of the esophagus. Am J Pathol 1999;155:1027–32.
23. Hao XP, Pretlow TG, Rao JS, Pretlow TP. β-Catenin expression is altered in human colonic aberrant crypt foci. Cancer Res 2001;61:8085–8.
24. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989. p. 463–9.
25. Jameson BA, Wolf H. The antigen index: a novel algorithm for predicting antigenic determinatants. Comput Appl Biosci 1988;4:181–6.
26. Velasco JA, Castro R, Avila MA, et al. cph, a novel oncogene which cooperates with H-ras in the transformation of NIH3T3 fibroblasts. Oncogene 1994;9:2065–9.
27. Briggs SL. The role of fibronectin in fibroblast migration during tissue repair. J Wound Care 2005;14:284–7.